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The Large First Periplasmic Loop of SecD and SecF Plays an Important Role in SecDF Functioning

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A remarkable feature of proteins of the SecD and SecF family involved in protein translocation is that they possess a very large first periplasmic domain. Here we report that this large first periplasmic domain is not required for the SecD-SecF interaction but that it is important for catalyzing protein translocation.

In bacteria, the majority of secretory proteins and membrane proteins are translocated across or inserted into the cytoplasmic membrane by the Sec machinery. In *Escherichia coli*, the Sec machinery comprises eight proteins, SecA, SecD, SecE, SecF, SecG, SecY, YajC, and YidC (see reference 2 for a review). SecY, SecE, and SecG form a heterotrimeric integral membrane domain with which a peripheral ATPase, SecA, associates to form the core of the translocase complex. SecD, SecF, and YajC form a distinct complex that can associate with the SecYEG complex (4). YidC has been discovered as an integral membrane protein that is in the close vicinity of transmembrane segments that insert into the membrane either independently or via the SecYEG complex (16, 18). YidC can be found associated to both the heterotrimeric SecYEG and SecDF-YajC complexes (12, 16). In analogy with OXA1 in mitochondria, YidC plays a crucial role in the biogenesis of respiratory chain complexes (19).

Reconstitution studies showed that SecY, SecE, and SecA are essential components of the Sec machinery and that SecG greatly enhances the reconstituted translocation activity (1, 7). Paradoxically, whereas cells lacking SecD and/or SecF are severely defective in protein export and barely viable (14), core constitution of SecDF (with or without YajC) with SecYEG does not further enhance the reconstituted translocation activity (10). Many roles for the SecDF-YajC complex in protein translocation have been postulated. Most importantly, this concerns a role of SecDF-YajC in regulating SecA cycling (5, 6) and in the release of preproteins from the translocase once they are translocated across the cytoplasmic membrane (9). The latter function might explain the remarkable feature of the SecD and SecF proteins, that they contain a very large first periplasmic loop (P1) that comprises about one-third of their total size. To study the importance of this periplasmic loop in SecDF functioning, we constructed *E. coli* SecD and SecF mutants with deletions in this loop. Here we show that already small deletions result in a protein with an impaired function, whereas it does not affect the stability of the SecDF-YajC complex.

In vivo analysis of SecD and SecF mutants with deletions in

the large periplasmic loop. To study the role of the very large first periplasmic domain (P1) in SecDF functioning, we created a set of in-frame deletions in *secD* and *secF* encoding this domain (Fig. 1, corresponding amino acid deletions). As SecD, SecF, and YajC are encoded by a single operon, which likely results in a well-balanced expression of the three proteins, we expressed the different deletion mutants from a plasmid containing the whole *yajC-secDF* operon under the control of an IPTG (isopropyl- α -D-thiogalactopyranoside)-inducible promoter. To test the functionality, plasmids containing the different deletions mutants were transformed to *E. coli* strain JP325 [*ara* Δ 714 Δ (*argF-lac*)U169 *rpsL150 relA1 thi flb5301 deoC1 ptsF25 recA::cat tgt::kan-araC*-P_{BAD}::*yajC-secDF* (6)]. In this strain, the chromosomal *yajC-secDF* operon is under the control of the *araB* promoter. Consequently, strain JP325 expresses only YajC, SecD, and SecF in the presence of arabinose and does not form colonies on LB agar plates in the presence of glucose. Growth on glucose-containing medium, however, can be restored by introducing a plasmid containing the *yajC-secDF* operon into the strain (the low basal expression from the *trc* promoter is sufficient to restore growth). Plasmids encoding SecD P1 deletion mutants very weakly restored growth of strain JP325 on glucose LB agar plates at 37°C (SecD Δ 199-279 and SecD Δ 33-151) or did not restore growth at all (SecD Δ 325-341 and SecD Δ 210-419). The weak complementation of mutants SecD Δ 199-279 and SecD Δ 33-151 became worse at lower temperatures. In contrast, the plasmid encoding the SecF P1 deletion mutant (SecF Δ 74-101) gave a strong cold-sensitive phenotype. It was perfectly able to restore growth of strain JP325 at 37°C but not at a temperature of 30°C or lower. The results described above did not change upon the overproduction of the different P1 deletion mutants by the addition of 300 μ M IPTG to the LB agar plates.

In vivo expression of the SecD and SecF deletion mutants. The introduction of large deletions in proteins may render them unstable. To determine if the different P1 deletion mutants form a stable protein in the membrane, we expressed them under similar conditions as we tested their ability to restore growth of strain JP325 on glucose medium. To this end, chromosomally encoded SecDF-YajC was depleted by growing strain JP325 for six generations on glucose-containing LB medium. Inner-membrane vesicles (IMVs) were isolated, and the expression of plasmid-encoded SecDF-YajC was analyzed by

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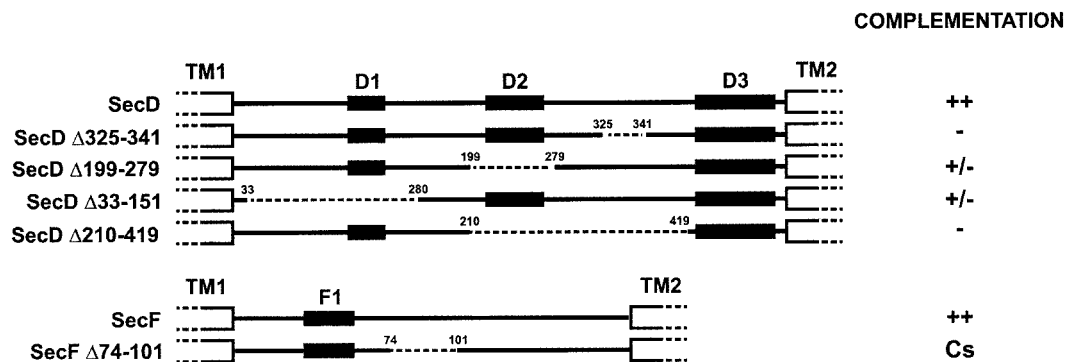


FIG. 1. Schematic representation of different P1 deletions in SecD and SecF and their ability to complement growth of the SecDF-YajC depletion strain JP325. Regions that are conserved are indicated by black boxes. To test the functionality of the deletion, strain JP325 ($P_{BAD}::yajC\text{-}secDF$) was transformed with plasmids pET545 (*yajC-secDF*), pNN230 (*yajC-secD* $\Delta 325-341F$), pNN231 (*yajC-secD* $\Delta 199-279F$), pNN232 (*yajC-secD* $\Delta 33-151F$), pNN233 (*yajC-secD* $\Delta 210-419F$), pNN235 (*yajC-secDF* $\Delta 74-101$), and analyzed to restore growth on LB-ampicillin (100 $\mu\text{g/ml}$) plates containing 0.5% glucose (with or without 300 μM IPTG) at different temperatures (37°C, 30°C, and 22°C). ++, growth; +/-, very poor growth; -, no growth; Cs, growth is cold sensitive (normal growth at 37°C and no growth at 30°C or lower).

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting by using antibodies against SecD and SecF. Using plasmid pET545 encoding wild-type SecDF-YajC, this procedure leads to near-chromosomal levels of SecD and SecF (13). Unfortunately, with the plasmids encoding P1 SecD/F deletion mutants, the expression levels of SecD and SecF obtained were lower than those from cells containing the wild-type plasmid (data not shown). These levels could be equalized by induction of the operon with 300 μM IPTG (Fig. 2, lanes 2 to 6). As a consequence, the final SecD and SecF levels in the membrane are higher than those from wild-type cells. As induction with IPTG did not affect the in vivo complementation results and different protein levels

would strongly interfere with the analysis of results from functional studies, we decided to use the membranes containing elevated SecD and SecF levels in the remainder of our study. Membranes from cells expressing SecD $\Delta 325-341$ and SecD $\Delta 210-419$ contained an ~ 60 -kDa protein and an ~ 40 -kDa protein, respectively (Fig. 2A, lanes 3 and 6). This corresponds well with the calculated molecular size of the corresponding deletion mutant. In contrast, IMVs from cells expressing SecD $\Delta 199-279$ (Fig. 2A, lane 4) or SecD $\Delta 33-151$ (Fig. 2A, lane 5) contain a protein that is much smaller than expected from the calculated molecular size. As both proteins reacted with antibodies against SecD (data not shown), the results suggest that these two SecD mutants are unstable.

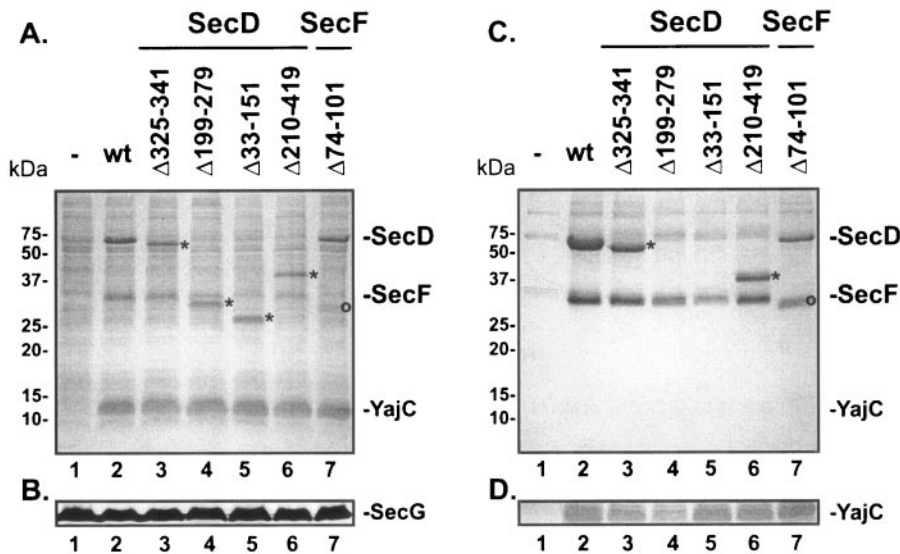


FIG. 2. Expression of P1 SecD/F deletion mutants in cells from which chromosomally encoded YajC-SecDF was depleted. IMVs were prepared from strain JP325 containing the different P1 deletion plasmids grown for six generations on LB medium containing 0.5% glucose. Expression of the plasmid-encoded gene products has been induced with 300 μM IPTG. IMVs were analyzed by SDS-PAGE and CBB staining (A) and by immunostaining using antibodies against SecG (B). (C) After solubilization of the IMVs with dodecylmaltoside, SecF was purified with Ni-NTA beads, and the copurification of SecD was analyzed by SDS-PAGE and CBB staining. (D) The presence of YajC in the Ni-NTA-purified material was analyzed by SDS-PAGE and silver staining. Positions of mutant SecD and SecF proteins are indicated with asterisks and circles, respectively. wt, wild type.

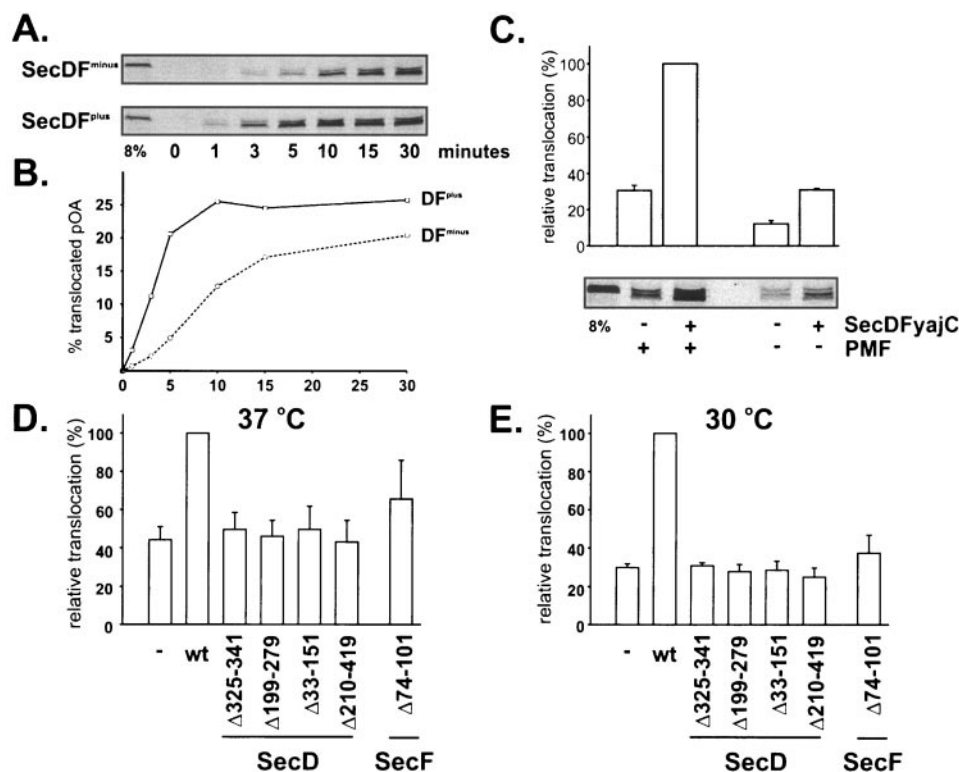


FIG. 3. SecDF-YajC-depleted IMVs have a kinetic translocation defect. (A) proOmpA translocation into IMVs derived from wild-type and SecDF-YajC-depleted cells (37°C). (B) Quantification results as shown in panel A. (C) proOmpA translocation into IMVs derived from wild-type and SecDF-YajC-depleted cells in the absence and presence of a PMF. IMVs containing P1 SecD/F deletion mutants are defective in protein translocation. (D and E) Initial rates of proOmpA translocation into IMVs containing different P1 SecD/F deletion mutants were analyzed at 37°C and 30°C. Translocation of proOmpA-fluorescein (4 μ g/ml) into IMVs containing the different mutant SecDF-YajC complexes (200 μ g/ml) were performed in 50 mM HEPES-KOH, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 0.2 mg/ml bovine serum albumin, 2 mM dithiothreitol with SecA (10 μ g/ml), SecB (35 μ g/ml), 10 mM phosphocreatine, 50 μ g/ml creatine kinase, 5 mM succinate, and 10 mM NADH. After the mixture was warmed for 2 min at 37°C, the reaction was initiated by the addition of ATP (2 mM final concentration). After the indicated period (A) or 10 (37°C [D])/15 (30°C [E]) minutes, samples were treated with proteinase K (0.1 mg/ml) for 30 min on ice. Translocation was assayed as described in reference 3. (B) To dissipate the PMF, nigericin and valinomycin (2 μ M final concentration) were added to the translocation mixture. (D and E) wt, wild type.

Membranes from cells expressing a mutant SecF with a 28-amino-acid deletion in the P1 loop contain normal levels of SecD and (mutant) SecF compared to the wild-type control (SecFΔ74-101 [Fig. 2A, lane 7]). It has been reported that SecDF-YajC depletion results in a twofold reduction of SecG (8). However, with our method to deplete chromosomally encoded SecDF-YajC while expressing the SecD and SecF deletion mutants, no changes in the SecG level were observed (Fig. 2B, compare lane 1 with lanes 2 to 7).

SecD and SecF deletion mutants form a stable SecDF-YajC complex. YajC, SecD, and SecF form a stable heterotrimeric complex in detergent solution (4, 12). To determine if the deletions in the P1 loop affect complex formation, IMVs overproducing the different P1 deletion mutants were solubilized with the detergent dodecylmaltoside, and the membrane extract was incubated with Ni-nitrilotriacetic acid (NTA) agarose beads. Under these conditions, wild-type YajC and SecD copurify with the histidine-tagged SecF (Fig. 2C and D, lanes 2) (12). The same copurification occurs with the membrane extract from cells expressing SecDΔ325-341, SecDΔ210-419, and SecFΔ74-101, indicating that these three mutants form a stable SecDF-YajC complex (Fig. 2C and D, lanes 3, 6, and 7). The unstable SecDΔ199-279 and SecDΔ33-151 mutants do not co-

purify with histidine-tagged SecF (Fig. 2C, lanes 4 and 5). Immunoblotting showed that YidC copurifies with both wild-type and mutant SecDF-YajC complexes (data not shown). However, the Coomassie blue-stained gel with the SecDΔ325-341 and SecDΔ210-419 mutants indicates that the YidC fraction that copurifies is small compared to the amount of SecD and SecF in the complex (Fig. 2C, lanes 3 and 6).

Inner-membrane vesicles containing SecD and SecF with a deletion in the large periplasmic loop display an *in vitro* translocation defect. Thus far, *in vitro* assays did not reveal a clear stimulatory effect of SecDF-YajC on protein translocation. We observed that when chemical amounts of proOmpA were used, the translocation kinetics into IMVs containing SecDF-YajC is about twofold faster than that into IMVs lacking SecDF-YajC (Fig. 3A). In the absence of a proton motive force (PMF), the total amount of proOmpA translocation is reduced, but still a twofold difference between SecDF-YajC-containing and -depleted IMVs is found (Fig. 3B). This further demonstrates that SecDF-YajC is not essential for proton motive force-driven protein translocation.

To test the effects of the deletions in the large periplasmic loop, we used the assay described above to determine the initial rates of proOmpA translocation at 37°C and 30°C. All

deletions resulted in a decrease in the rate of proOmpA translocation. For both temperatures and for all deletions in the periplasmic P1 loop of SecD, this decrease is similar to the reduction observed with IMVs that lack the SecDF-YajC complex (Fig. 3C and D). proOmpA translocation into IMVs from SecFA74-101 mutant cells shows a cold-sensitive phenotype. Although at 37°C proOmpA translocation into SecFA74-101 IMVs is lower than that for wild-type IMVs, translocation is clearly better than that for SecDF-YajC-depleted IMVs. The latter difference, however, is nearly diminished at a low temperature, i.e., 30°C. To exclude the possibility that the obtained translocation defects are caused by a reduced PMF generation, we measured PMF generation with ATP and NADH. With both energy sources, all IMVs were equally active in generating a PMF (data not shown). Thus, the reduced translocation into IMVs from the different mutants is directly caused by the deletion in the P1 loop.

Our study shows that small deletions in P1-SecD and P1-SecF (13 and 28 amino acids, respectively) impaired the SecDF functioning both *in vivo* and *in vitro*. As both mutant proteins are expressed and are able to form a stable SecDF-YajC complex, this demonstrates that the periplasmic P1 domain in SecD and SecF fulfills a critical function. Moreover, since a mutant SecD with a large deletion in P1 (SecD Δ 210-419) still forms a complex with SecF and YajC (Fig. 2C, lane 6), the large periplasmic P1 domain is likely not important for the interactions within the SecDF-YajC complex but plays a main role in catalyzing protein translocation.

What could be the role of the large periplasmic P1 domain in SecDF functioning? First, SecD and SecF are members of the large superfamily of RND proteins (17). These membrane proteins contain 12 (or 2 times 6) transmembrane segments (TMs) with large periplasmic domains between TMs 1 and 2 and between TMs 7 and 8. The crystal structure of AcrB (11), one of the members of the RND superfamily, showed that the large periplasmic domains form the basis of a chamber-like structure in the periplasm. In the case of the SecDF-YajC complex, the two large periplasmic domains might form a similar structure. Such a structure could create a protected surrounding in the periplasm in which proteins can fold after they are translocated by the SecYEG complex. The overall effect would be that proteins fold more efficiently at the periplasmic side of the membrane, while their release from the transport apparatus would be controlled (9). Second, the large periplasmic domains may interact with other components in the cell. For instance, it has been shown that SecF stabilizes SecY (15). The large first periplasmic domain in SecF might be responsible for the interaction with SecY. The interaction of the SecDF-YajC complex with SecYEG may, for instance, serve to facilitate oligomerization of SecYEG protomers, thereby stabilizing the active translocation channel. Future studies should address these options in more detail.

Thus far, *in vitro* translocation studies did not reveal differences between IMVs containing and lacking SecDF-YajC. In this study, we showed that the kinetics of proOmpA translocation in IMVs lacking SecDF-YajC is twofold slower than that in IMVs containing SecDF-YajC. The difference in kinetics becomes larger at lower temperatures (data not shown). This shows that, like the case *in vivo*, the SecDF-YajC complex also has a catalytic function *in vitro* which is sensitive to cold.

However, to detect the activity of SecDF-YajC *in vitro*, it is very important to perform kinetic studies using chemical amounts of substrates in the reaction mixture. This explains why the *in vitro* catalytic function of the SecDF-YajC complex was not observed previously; as in other studies, radiochemical amounts of substrates and/or long incubation times were used (4, 8). This assay, along with the availability of inactive mutants that form a stable SecDF-YajC complex, is a good starting point to develop an *in vitro* assay that is based on liposomes containing purified SecYEG and SecDF-YajC. With such an assay, the catalytic function of the SecDF-YajC complex in protein translocation could be analyzed in more detail.

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